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Table of Contents

Page

INTRODUCTION

We propose that MIS regulation may be an important element contributing to the ability of 1,25-dihydroxyvitamin D_3 (calcitriol) to inhibit the growth and progression of prostate cancer cells. We have shown that calcitriol acts by several pathways to inhibit the growth of prostate cancer cells [1-5]. In the current studies we show that calcitriol also stimulates the expression of MIS in a standard human prostate cancer cell line, LNCaP. We further show that the up-regulation of MIS expression is mediated directly by calcitriol binding to the vitamin d receptor (VDR) and the complex subsequently interacting with a vitamin D regulatory element (VDRE) in the MIS gene promoter.

BODY

We have shown that in the LNCaP prostate cancer cell line, calcitriol treatment upregulates MIS gene expression and MIS protein levels. We identified a 15 bp sequence GGGTGAgcaGGGACA at –395/-381 from the ATG start site in the MIS promoter (Fig.1). The MIS promoter also contains response lements for GATA-4, SOX9 and SF-1.

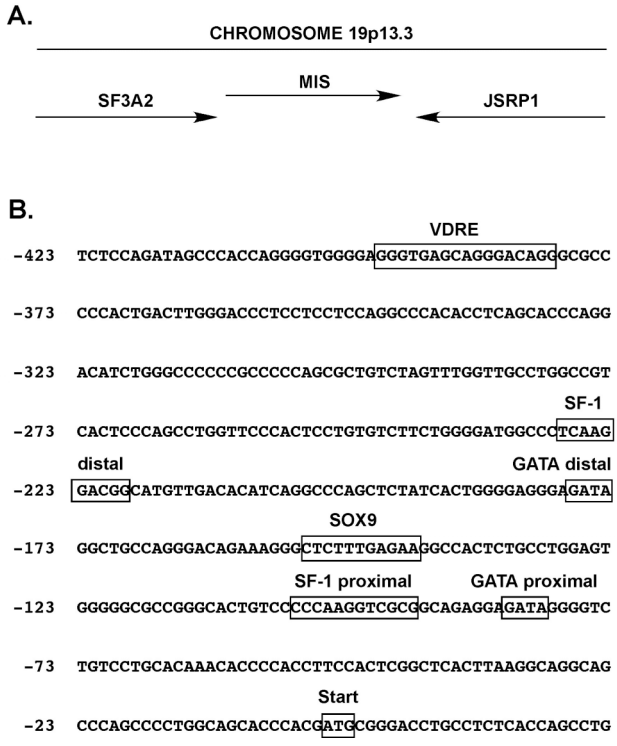

Fig. 1. A putative vitamin D response element (VDRE) is present in the MIS promoter. Panel A: The human MIS gene is on chromosome 19 and is located between the SF3A2 gene and the JSRP1 gene. The MIS transcriptional start site is located only 748 bp downstream of the termination codon of the SF3A2 gene. Arrows indicate direction of transcription. Panel B: Using *in silico* analysis, we identified a putative VDRE in the MIS promoter. The VDRE is located at

nucleotides –381 to –396 relative to the ATG translation start site. The location of transcription factor binding sites for SF-1, SOX9 and GATA-4 that regulate MIS promoter activity are also shown.

We cloned a 680 bp fragment (-657 to +23) of the MIS promoter containing the putative VDRE into a luciferase reporter vector (Fig. 2). In transactivation assays in HeLa cells, we demonstrated that the MIS promoter activity was upregulated by calcitriol.

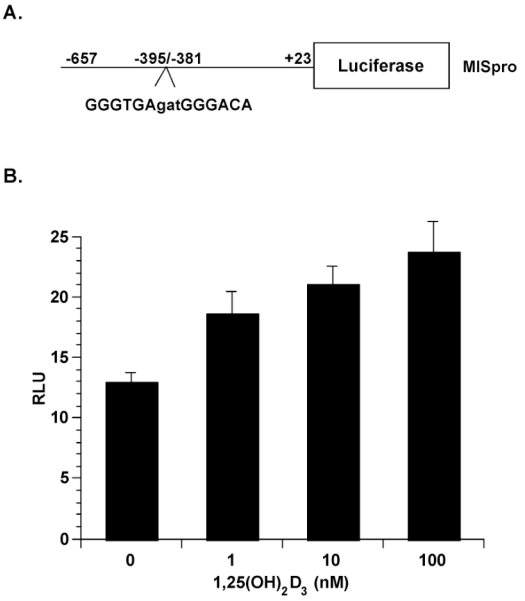

Fig. 2. Transactivation of the MIS promoter by 1,25-$(OH)_2D_3$ in HeLa cells. Panel A: Illustration of the MIS promoter construct. Panel B: HeLa cells were transfected with a VDR expression vector and the MIS promoter luciferase reporter construct. Cells were treated with vehicle or 1,25-$(OH)_2D_3$ for 24 hr. Luciferase activity was measured using the dual luciferase assay. Values represent mean ±SD of triplicate transfections.

Co-expression of VDR with steroidogenic factor 1 (SF-1), a key regulator of MIS, increased MIS promoter basal activity that was further stimulated by calcitriol treatment (Fig. 3).

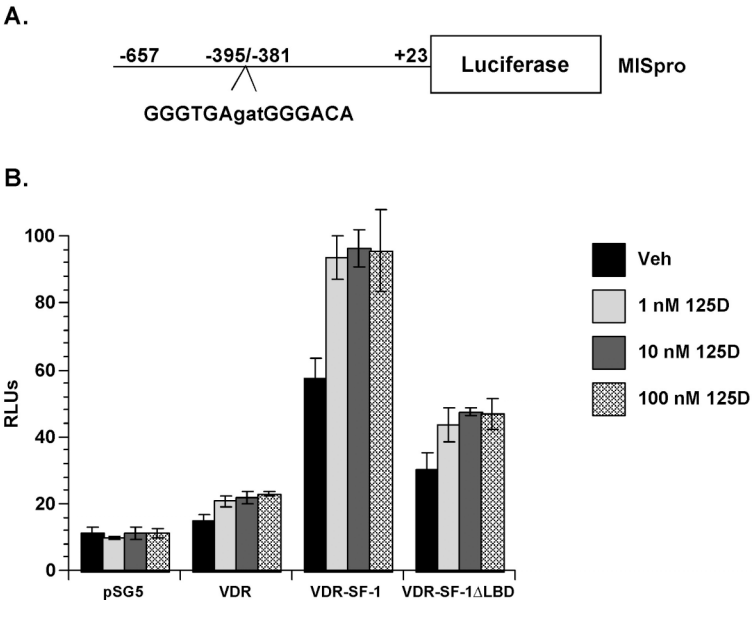

Fig. 3. Steroidogenic factor 1 (SF-1) and VDR cooperate to increase MIS promoter activity in response to 1,25-$(OH)_2D_3$. Panel A: MIS promoter-luciferase construct. Panel B: Transactivation assays in HeLa cells transfected with vector alone (pSG5), VDR alone, VDR and SF-1 or VDR and SF-1 with a deletion of the ligand binding domain (SF-1ΔLBD) and the MIS promoter-luciferase construct. Cells were

treated with vehicle or 1,25-$(OH)_2D_3$ for 24 hr and luciferase activity measured. Values represent mean ±SD of triplicate transfections.

We created a single point mutation in the putative VDRE or deleted of the entire 15 base sequence. Both mutations resulted in the loss of calcitriol-induced transactivation (Fig. 4).

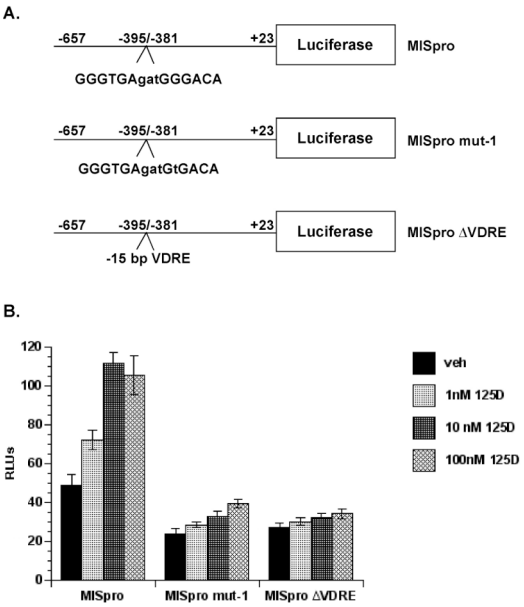

Fig. 4. Mutations in the MIS VDRE reduce 1,25-$(OH)_2D_3$ responsiveness. Panel A: A single G to T point mutation in the 3-prime hexamer of the VDRE (MISpro mut-1) and a 15 bp deletion of the entire VDRE (MISpro ΔVDRE) were created in the MIS promoter. Panel B: Transactivation assays in HeLa cells transfected with VDR and SF-1 expression vectors and the MIS promoter luciferase constructs. Cells were treated with vehicle or 1,25-$(OH)_2D_3$ for 24 hr and luciferase activity measured. Values represent mean ±SD of triplicate transfections.

In gel shift assays, the VDR bound the MIS VDRE and the binding was increased by calcitriol (Fig. 5).

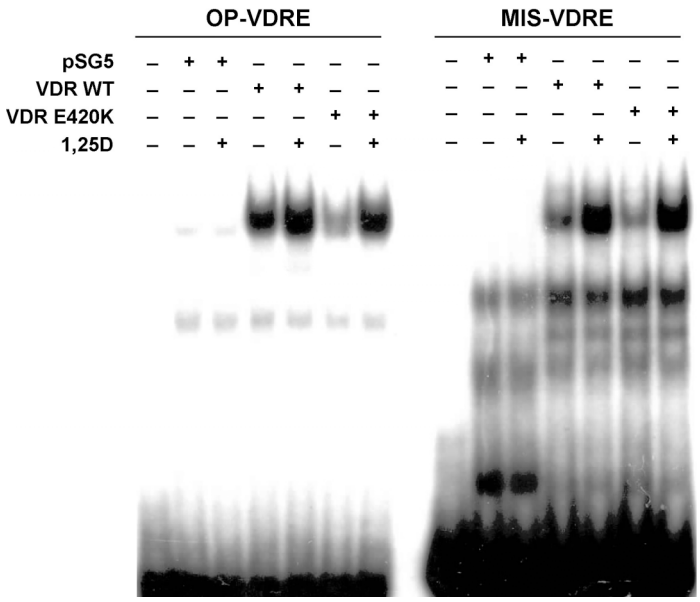

Fig. 5. The VDR binds to the MIS VDRE *in vitro*. [^{32}P]-labeled MIS and osteopontin (OP) VDREs were incubated with COS-7 extracts transfected with vector alone (pSG5), WT VDR, or VDR E420K a coactivator binding defective mutant. Samples were incubated with and without 10 nM 1,25-$(OH)_2D_3$ for 30 min at ambient temperature. The samples were then

electrophoresed on 5% polyacrylamide gels in 0.5X Tris-borate buffer for 170 min at 70 volts. Bands were visualized by autoradiography. 1,25D, 1,25-$(OH)_2D_3$.

We cloned the 15 bp MIS VDRE sequence into an enhancerless heterologous promoter in pGL3promoter vector. The 15 bp MIS VDRE was able to confer vitamin D responsiveness to the heterologous promoter demonstrating that this nucleotide sequenced is a bona fide VDRE (Fig. 6).

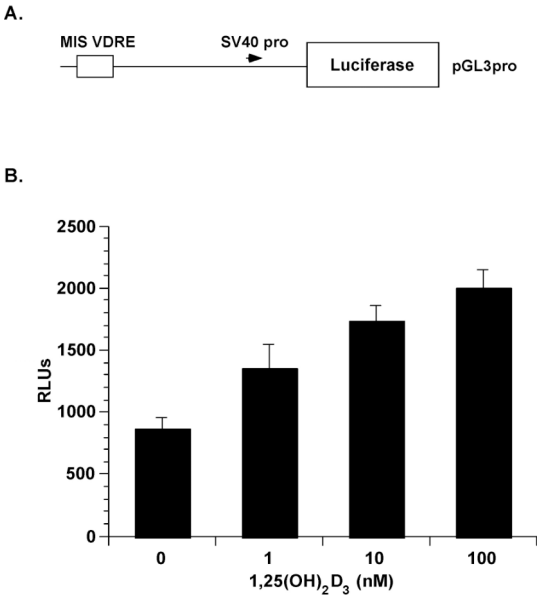

Fig. 6. The MIS VDRE confers 1,25-$(OH)_2D_3$ responsiveness to a heterologous promoter. Panel A: The 15 bp MIS VDRE was cloned into pGL3-promoter containing an SV40 promoter. Panel B: Transactivation assays in HeLa cells transfected with a VDR expression vector and the MIS VDRE luciferase reporter construct. Cells were treated with vehicle or 1,25-$(OH)_2D_3$ for 24 hr and luciferase activity measured. Values represent mean ±SD of triplicate transfections.

In Fig. 7, we demonstrate *in vivo* that the VDR binds to the MIS promoter in LNCaP cells using chromatin immunoprecipitation.

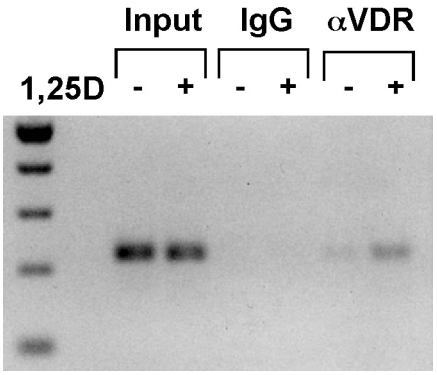

Fig. 7. The VDR interacts with the MIS VDRE in intact LNCaP human prostate cancer cells. LNCaP cells were treated with and without 10 nM 1,25-$(OH)_2D_3$ for 6 hr at 37°C. The cells were then fixed with 1% formaldehyde for 10 min at ambient temperature. The samples were washed with phosphate-buffered saline and then analyzed by chromatin immunoprecipitation (ChiP) assays. ChiP assays were performed with VDR and control IgG antibodies. Input represents DNA extracted prior to ChiP assay.

We have demonstrated for the first time that the MIS gene is a new target of calcitriol action. The MIS promoter contains a functional VDRE that binds the VDR and MIS is upregulated by calcitriol in prostate cancer cells. We are currently determining whether upregulation of MIS contributes to the anti-proliferative/pro-apoptotic actions of calcitriol in prostate cancer. We have recently obtained purified MIS from Dr. Donahoe's lab that we will use to test the combined effects of calcitriol and MIS on prostate cancer cell growth.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that MIS is produced by human prostate cancer cells
- Proved that MIS is a target of calcitriol
- Identified a VDRE in the MIS promoter
- Confirmed in vivo in cells that VDR acting via the VDRE up-regulates MIS

REPORTABLE OUTCOMES

P.J. Malloy and D. Feldman. Mullerian Inhibitory Substance (MIS) is upregulated by 1,25-dihydroxyvitamin D_3 in LNCaP prostate cancer cells via a direct interaction of the vitamin D receptor with a vitamin D regulatory element in the MIS promoter.
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CONCLUSIONS

These studies add a new dimension to calcitriol action by adding the regulation of a new and previously unknown protein to the list of regulated substances by calcitriol. Since MIS is an important protein that regulates organ development and is currently in studies as an anti-cancer agent, this finding suggests that MIS amy be useful in prostate cancer therapy and that calcitriol's regulation of MIS may contribute to its anti-cancer activity. Future studies will examine whether adding MIS to calcitriol will enhance the action of both molecules as anti-cancer agents.

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